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Microfluidic Quantitative PCR for Simultaneous Quantification of Multiple Viruses in Environmental Water Samples

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To secure food and water safety, quantitative information on multiple pathogens is important. In this study, we developed a microfluidic quantitative PCR (MFQPCR) system to simultaneously quantify 11 major human viral pathogens, including adenovirus, Aichi virus, astrovirus, enterovirus, human norovirus, rotavirus, sapovirus, and hepatitis A and E viruses. Murine norovirus and mengovirus were also quantified in our MFQPCR system as a sample processing control and an internal amplification control, respectively. River water contaminated with effluents from a wastewater treatment plant in Sapporo, Japan, was collected and used to validate our MFQPCR system for multiple viruses. High-throughput quantitative information was obtained with a quantification limit of 2 copies/μl of cDNA/DNA. Using this MFQPCR system, we could simultaneously quantify multiple viral pathogens in environmental water samples. The viral quantities obtained using MFQPCR were similar to those determined by conventional quantitative PCR. Thus, the MFQPCR system developed in this study can provide direct and quantitative information for viral pathogens, which is essential for risk assessments.

Food- and waterborne viruses can cause a number of human diseases. Norovirus (NoV) is the major cause of diarrhea in both children and adults (1), and rotavirus (RoV) is the leading cause of hospitalizations for diarrhea among children younger than 5 years (2). In addition to gastroenteritis, some waterborne viruses, such as hepatitis A virus (HAV) and hepatitis E virus (HEV), can cause human hepatitis via fecal-oral transmission (3, 4). Food and water contamination by these and other viral pathogens has caused disease outbreaks even in developed countries with drinking water and wastewater treatment systems (1, 5, 6). For example, NoV outbreaks occurred through drinking water in Finland (7) and in New Zealand (8). Thus, to decrease the risks of viral infection and to prevent disease outbreaks, it is important to detect and quantify these viral pathogens in food and water samples.

Quantitative PCR (qPCR) and its derivative, reverse transcription-qPCR (RT-qPCR), have been widely used to detect and quantify viral pathogens in food and water samples because, to date, qPCR is the most sensitive and specific method available (9). Numerous qPCR and RT-qPCR assays have been developed to quantify viral pathogens, including NoV (10), RoV (11), HAV (12), and HEV (13). However, most of these assays can target only one pathogen per assay. Therefore, many qPCR or RT-qPCR runs are required to quantify multiple pathogenic viruses. Quantification of several target molecules in a single reaction can be achieved by multiplex qPCR with TaqMan probes that are labeled with different fluorophores (14–17). However, with current qPCR instruments, only 2 to 5 fluorophores can be differentiated, which limits the number of targets that can be simultaneously quantified.

We previously developed a system that could simultaneously quantify multiple enteric bacteria in environmental samples by using microfluidic quantitative PCR (MFQPCR) technology (18). With this MFQPCR system, multiple singleplex TaqMan qPCR assays are run in parallel in nanoliter chambers that are present at a high density on a single chip. This MFQPCR system was successfully applied to quantitatively detect multiple pathogens in a natural freshwater lake that was seasonally contaminated by waterfowl feces (19). Pathogen concentrations obtained with this system could then be used for quantitative microbial risk assessment (QMRA) (19). Several advantages of this MFQPCR over other simultaneous multipathogen detection technologies such as microarray (20, 21), TaqMan array (22, 23), Luminex assay (24), OpenArray (25), FilmArray (26), and molecular inversion probe assay (27) include its high sensitivity and quantitative performance. However, MFQPCR technology has not been applied to quantify multiple viral pathogens.

Consequently, the objectives of this study were to (i) develop an MFQPCR system to quantify multiple pathogenic viruses and (ii) apply this method for quantifying pathogenic viruses in environmental samples. We targeted major food and waterborne human viruses, including adenovirus (AdV) types 40 and 41, Aichi virus (AiV), astrovirus (AsV), enterovirus (EV), NoV genogroup I (GI), GII, and GIV, RoV group A, sapovirus (SaV) GI, GII, GIV, and GV, HAV, and HEV. In addition, mengovirus (MgV) and murine norovirus (MNV) were used as control viruses.

MATERIALS AND METHODS

Concentration of viral particles from water samples. Environmental water samples (n = 32) were collected from the Motsukisamu River (43.0699°N, 141.4196°E) in Sapporo, Japan, from December 2011 to April 2013. The sampling site was located downstream from a wastewater treatment plant.

Viral particles in water samples were concentrated by a negatively

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TABLE 1 Primers and probes used in this study

Target(s)	Name	Sequence (5′→3′)[a]	Reference
Adenovirus types 40 and 41	JTVXF	GGACGCCTCGGAGTACCTGAG	31
	JTVXR	ACIGTGGGGTTTCTGAACTTGTT	
	JTVXP	FAM-CTGGTGCAG/ZEN/TTCGCCCGTGCCA-IBFQ	
Aichi virus	AiV-AB-F	GTCTCCACHGACACYAAYTGGAC	32
	AiV-AB-R	GTTGTACATRGCAGCCCAGG	
	AiV-AB-TP	FAM-TTYTCCTTYGTGCGTGC-NFQ-MGB	
Astrovirus	AV1	CCGAGTAGGATCGAGGGT	33
	AV2	GCTTCTGATTAAATCAATTTTAA	
	AVS	FAM-CTTTTCTGT/ZEN/CTCTGTTTAGATTATTTTAATCACC-IBFQ	
Enterovirus	Ev2	CCCCTGAATGCGGCTAATC	34
	Ev1	GATTGTCACCATAAGCAGC	
	Ev-probe	FAM-CGGAACCGA/ZEN/CTACTTTGGGTGTCCGT-IBFQ	
Human norovirus GI	NIFG1F	ATGTTCCGCTGGATGCG	10
	NV1LCR	CCTTAGACGCCATCATCATTTAC	
	NIFG1P	FAM-TGTGGACAG/ZEN/GAGAYCGCRATCT-IBFQ	
Human norovirus GII	NIFG2F	ATGTTCAGRTGGATGAGRTTCTC	10
	COG2R	TCGACGCCATCTTCATTCACA	
	QNIFS	FAM-AGCACGTGG/ZEN/GAGGGCGATCG-IBFQ	
Human norovirus GIV	NIFG4F	ATGTACAAGTGGATGCGRTTC	10
	COG2R	TCGACGCCATCTTCATTCACA	
	NIFG4P	FAM-AGCACTTGG/ZEN/GAGGGGGATCG-IBFQ	
Hepatitis A virus	HAV68	TCACCGCCGTTTGCCTAG	12
	HAV240	GGAGAGCCCTGGAAGAAAG	
	HAV150(-)	FAM-CCTGAACCTGCAGGAATTAA-NFQ-MGB	
Hepatitis E virus	HECOM-S	CGGCGGTGGTTTCTGGRGTG	13
	HECOM-AS	GGGCGCTKGGMYTGRTCNCGCCAAGNGGA	
	TP-HECOM	FAM-CCCCYATAT/ZEN/TCATCCAACCAACCCCTTYGC-IBFQ	
Rotavirus A	Rota-NVP3-F	ACCATCTACACATGACCCTC	11
	Rota-NVP3-R	GGTCACATAACGCCCC	
	Rota-TaqMan	FAM-ATGAGCACA/ZEN/ATAGTTAAAAGCTAACACTGTCAA-IBFQ	
Sapovirus GI, GII, GIV, and GV	SaV124F	GAYCASGCTCTCGCYACCTAC	35
	SaV1F	TTGGCCCTCGCCACCTAC	
	SaV5F	TTTGAACAAGCTGTGGCATGCTAC	
	SaV1245R	CCCTCCATYTCAAACACTA	
	SaV124TP	FAM-CCRCCTATRAACCA-NFQ-MGB	
	SaV5TP	FAM-TGCCACCAATGTACCA-NFQ-MGB	
Mengovirus	Mengo110	GCGGGTCCTGCCGAAAGT	36
	Mengo209	GAAGTAACATATAGACAGACGCACAC	
	Mengo147	FAM-ATCACATTACTGGCCGAAGC-NFQ-MGB	
Murine norovirus	MNV-S	CCGCAGGAACGCTCAGCAG	30
	MNV-AS	GGYTGAATGGGGACGGCCTG	
	MNV-TP	FAM-ATGAGTGATGGCGCA-NFQ-MGB	

[a] FAM, 6-fluorescein amidite; NFQ-MGB, nonfluorescent quencher with a minor grove binder (Applied Biosystems); ZEN, ZEN internal quencher (Integrated DNA Technologies); IBFQ, Iowa Black fluorescent quencher (Integrated DNA Technologies).

charged membrane method (28). To assess the efficiency of recovery of viral RNA during sample processing (29), MNV strain S7-PP3, which was kindly provided by Yukinobu Tohya (Nihon University) and was prepared using RAW 264.7 cells (ATCC TIB-71) as previously described (30), was added as a process control virus to the water samples. In brief, 10 μl of known concentrations of MNV (ranging from 2.9×10^7 and 8.2×10^9 copies/liter) were added to water samples (1 liter) along with 25 mM $MgCl_2$ and then filtered through negatively charged mixed cellulose ester membranes with a 0.45-μm pore size and 90-mm diameter (Millipore). After rinsing the membrane with 200 ml of 0.5 mM H_2SO_4 (pH 3.0), viral particles were eluted from the membrane with 10 ml of 1 mM NaOH (pH 10.8). The viral concentrates were neutralized upon elution with 0.1 ml of 50 mM H_2SO_4 and 0.1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Nucleic acid extraction and cDNA synthesis. DNA and RNA were coextracted from a 1-ml portion of the viral concentrates using NucliSENS magnetic extraction reagents (bioMérieux), according to the manufacturer's instructions, to a final elution volume of 110 μl. Aliquots (2 μl) were used for a reverse transcription (RT) reaction using PrimeScript RT reagent (TakaraBio) with 30 μM random 6-mers and 10 μM oligo(dT) primers in a total volume of 10 μl. Because we did not perform DNase treatment prior to the RT reaction, the resulting cDNA samples contained viral DNA from the original samples; therefore, we could detect both DNA and RNA viruses. cDNA samples were stored at −20°C until use.

Primers, probes, and plasmids for qPCR. Previously validated TaqMan qPCR assays (10–13, 30–36) were used for this study (Table 1). These assays have been successfully applied to specifically quantify viruses in water and other environmental samples. Using these assays, we could quantitatively detect 13 viruses, including AdV types 40 and 41, AiV, AsV, EV, NoV GI, GII, and GIV, RoV group A, SaV GI, GII, GIV, and GV, HAV (all genotypes), HEV (all genotypes), MgV, and MNV. Short TaqMan probes (<20 bp) were labeled with 6-fluorescein amidite (6-FAM) at their 5′ ends and a nonfluorescent quencher with a minor grove binder (MGB)

at their 3′ ends (synthesized by Applied Biosystems). Long TaqMan probes were labeled with 6-FAM at their 5′ ends, Iowa Black fluorescent quencher at their 3′ ends, and an internal ZEN quencher that was inserted between the 9th and 10th bases from their 5′ ends (synthesized by Integrated DNA Technologies).

Linearized plasmids that included the target gene sequences (Table 1) were synthesized or prepared as described previously (18). DNA concentrations were determined using PicoGreen double-stranded DNA (dsDNA) quantification reagent (Molecular Probes). Serial dilutions (10^0 to 10^6 copies/μl) of a mixture of the 13 plasmid DNA were used to generate standard curves for MFQPCR and conventional qPCR.

Conventional qPCR. Conventional TaqMan real-time qPCR was done using an ABI Prism 7500 Fast Sequence detection system (Applied Biosystems). The reaction mixture (10 μl) contained 2× FastStart universal probe master mix with ROX (Roche), 400 nM each forward and reverse primer, 200 nM TaqMan probe, and 1 μl of template DNA/cDNA. qPCR was run in duplicate using the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The results were analyzed using ABI Prism 7500 SDS software (Applied Biosystems).

MFQPCR. To increase the amount of target genes prior to MFQPCR, we used a specific target amplification (STA) reaction, a 14-cycle multiplex PCR, as described previously (18, 37) with the 28 primers (0.2 μM each) listed in Table 1. The STA reaction is necessary when small amount of target molecules is to be quantified by MFQPCR. In MFQPCR platform, qPCR is performed in 6.7-nl chamber; therefore, at least 1 copy/6.7 nl (= 150 copies/μl) of qPCR mixture is necessary. To obtain reliable quantitative results, it is better to use $>10^4$ copies/μl DNA/cDNA (38), which is generally too high for environmental samples.

The STA reaction mixture (10 μl) contained TaqMan PreAmp master mix (Applied Biosystems), 0.2 μM each primer, and 2.5 μl of the DNA/cDNA template. Both environmental DNA/cDNA samples and the standard plasmid mixture were subjected to the STA reaction. In the case of environmental DNA/cDNA samples, the 2.5-μl DNA/cDNA template was composed of 0.5 μl plasmid DNA that contained MgV gene sequences (2×10^4 copies/μl) and 2.0 μl of the DNA/cDNA samples. MgV plasmid DNA (10^4 copies) was added as an internal amplification control (IAC) to assess the presence of PCR inhibitors in the environmental DNA/cDNA samples. The STA reaction was performed using a Veriti 96-well thermal cycler (Applied Biosystems) under the following conditions: 95°C for 10 min, followed by 14 cycles of 95°C for 10 s and 60°C for 4 min. The STA products were diluted 6-fold with TE buffer for MFQPCR. Unbiased amplification by the STA reaction was verified by comparing the standard curves generated by conventional qPCR with DNA templates before and after the STA reaction. For this purpose, STA products diluted 60-fold with TE buffer were used as templates for qPCR.

MFQPCR was run in quadruplicate using a BioMark HD reader with a Dynamic Array 96.96 chip (Fluidigm), as previously described in detail (18). Singleplex TaqMan real-time qPCR was run in 6.7-nl chambers on a chip using 1× TaqMan Universal PCR master mix (Applied Biosystems), 400 nM (each) forward and reverse primers, and 200 nM (100 nM each for SaV) probe. qPCR was run using the following conditions: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s, 70°C for 5 s, and 60°C for 1 min. The results were analyzed using Real-Time PCR Analysis software version 3.0.2 (Fluidigm).

Data analysis. Standard curves were generated using simple linear regression for the quantification cycle (Cq) values versus the amounts of template DNA (log copies/μl). For environmental samples, the quantity of a target gene was calculated from the Cq values (with 2 to 4 replicates) using the standard curves. Sample recovery efficiencies were determined as the quantity of MNV measured by MFQPCR divided by the quantity of MNV spiked to the water samples. Occurrence of PCR inhibition was assessed by the IAC recovery efficiency as determined by the quantity of IAC measured divided by the quantity of IAC added prior to an STA reaction. Pearson correlation coefficients were determined using R version 3.0.2.

The quantification limit of the target molecules in each qPCR assay (QL_{assay}) was determined based on the amplification of the lowest concentration of the standard plasmid DNA. The quantification limit of the target molecules in environmental water samples (QL_{env}) was calculated by multiplying the QL_{assay} by the concentration factors given by each sample processing.

RESULTS AND DISCUSSION

Sensitivities of the qPCR assays. In this study, we used previously validated TaqMan qPCR assays. Some of these assays use TaqMan probes labeled with MGB, which can increase the melting temperature of the probes and reduce background fluorescent signals (39). Low background fluorescence is important for sensitive gene quantification with the MFQPCR platform. To reduce the background fluorescent signals in TaqMan qPCR assays with non-MGB probes, we used an internal ZEN quencher, which was previously used for nanoliter-scale fluorescence detection assays (40, 41). As a result, we could quantify all genes with high sensitivity, as low as 2 copies/μl (Fig. 1), using the MFQPCR platform.

To lower the quantification limit further, we ran STA reactions to preamplify target DNA molecules. When qPCR was run after the STA reaction, the Cq values were approximately 5 to 7 cycles smaller than those obtained from the qPCR done without the STA reaction (Fig. 1). This suggested that the amount of target DNA molecules had increased approximately 2^{11}- to 2^{13}-fold with the STA reaction, taking account of the 60-fold (ca. 2^6-fold) dilutions of the STA products prior to qPCR. In addition, quantitative performances (e.g., PCR efficiencies and linear dynamic ranges) were similar for the qPCRs done with and without STA reactions for almost all viruses. Means ± standard deviations (SD) of the PCR efficiencies were 106% ± 11% and 108% ± 14% for qPCR done with STA and without STA, respectively. We could quantify target molecules from 20 to 2×10^6 copies/μl in most of the qPCR assays. In many cases, we could detect lower concentrations of target molecules (i.e., 2 copies/μl), especially when qPCR was performed after an STA reaction. These results suggested that the STA reaction effectively increased the target gene copy numbers without any major effects on the qPCR quantitative performance, similar to the results in a previous study (18, 37).

Simultaneous quantification of multiple pathogenic viruses in environmental water samples. The MFQPCR system developed in this study was applied to quantifying multiple pathogenic viruses in river water samples that were contaminated by effluents from a wastewater treatment plant. We detected NoV GI and GII and RoV in 16%, 35%, and 23% of the water samples ($n = 32$), particularly during the winter months (December to March) (Fig. 2A). Similar to the results in this study, norovirus and rotavirus numbers increased during the winter in river water (42) and in wastewater (43, 44). Average (± SD) concentrations of NoV GI and GII and RoV in winter were 4.34 ± 0.33, 4.35 ± 0.31, and 4.47 ± 0.48 log copies/liter water, respectively, which were similar to the previously reported values in river water (42). Other pathogenic viruses were below the quantification limit (QL_{env} = 3.84 $\log_{10}$/liter water) of our MFQPCR system. The quantification limit of our qPCR assays in the MFQPCR system (QL_{assay}) was, in most cases, 2 copies/reaction; however, many steps during sample processing (viral concentration, RNA extraction, and cDNA synthesis) resulted in the relatively high QL_{env} values. More viruses

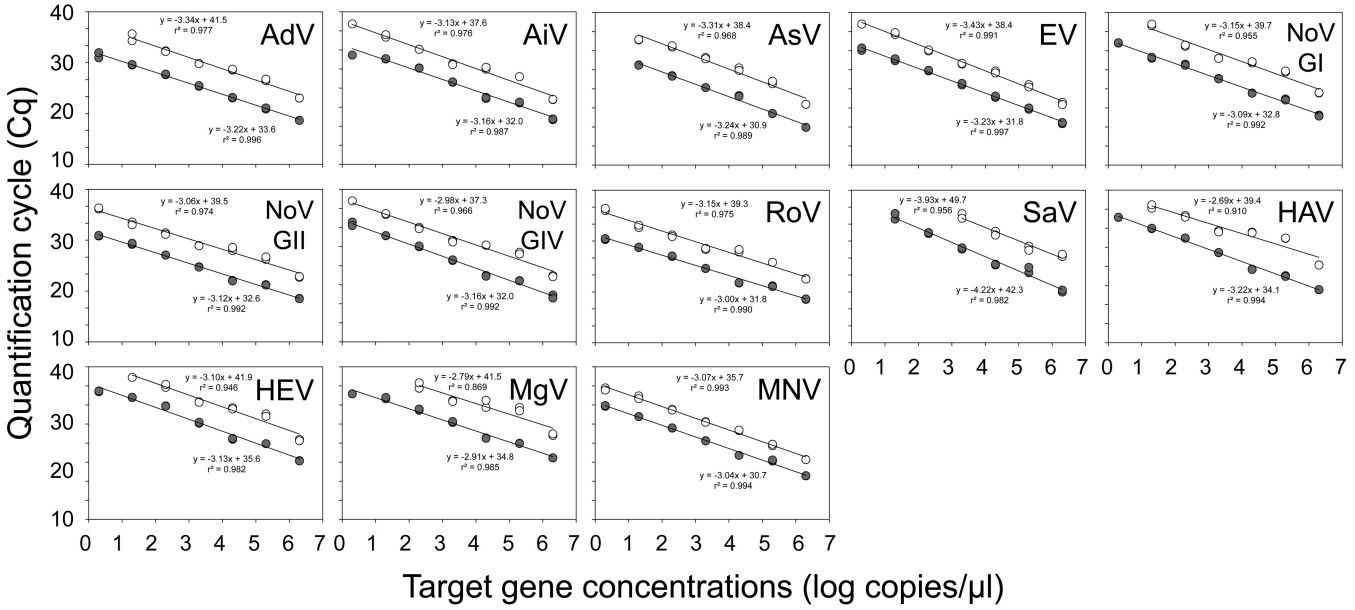

FIG 1 Standard curves generated based on the results of the qPCR done with STA reactions (●) and without STA reactions (○). The linear regression equations and goodness-of-fit (r^2) values are also shown for each assay.

might be detected if we could lower the quantification limit by increasing the volume of water filtered (see, e.g., reference 45).

MNV and MgV were detected in all samples (Fig. 2B). Both MNV and MgV have been used as process control viruses to determine sample recovery efficiencies (14, 36). In this study, MNV was used to determine sample recovery efficiencies, while MgV plasmid DNA (IAC) was used to determine IAC recovery efficiencies to assess the occurrence of inhibition during STA and qPCRs, although it is possible to reverse their roles (i.e., with MgV used as a sample process control [SPC] and MNV plasmid DNA used as an IAC). Sample recovery efficiencies and IAC recovery efficiencies fluctuated from 0.04% to 69.5% (mean, 9.4%) and from 1.2% to 38.7% (mean, 15.4%), respectively, during the sampling period. These results indicate that the sample recovery efficiencies and inhibitory effects in STA and qPCR varied greatly between samples. There was a positive correlation between the sample recovery efficiencies and the IAC recovery efficiencies ($r = 0.40$; $P < 0.05$). This indicated that the low sample recovery efficiency was probably due, in part, to inhibition during STA and qPCRs, which was similar to a previous study that used genetically engineered *Escherichia coli* as a sample processing control (46). Dilution or purification of RNA samples can effectively overcome PCR inhibition; however, these procedures can also decrease the total amount of RNA, which could influence the detection of target viruses that are at low concentrations. In fact, when RT and qPCR was performed with 10-fold-diluted RNA samples, sample recovery efficiencies were improved from 9.4% to 27.0%, whereas NoV GI and GII and RoV dropped below the quantification limit (data not shown). Regardless, the sample process control and IAC are useful for evaluating the performance for virus concentration, RNA extraction and purification, RT, STA, and qPCR (18, 29, 47). We can determine if any of these processes need to be redone based on low sample recovery efficiencies or IAC recovery efficiencies. Thus, inclusion of these controls in our MFQPCR system is an advantage.

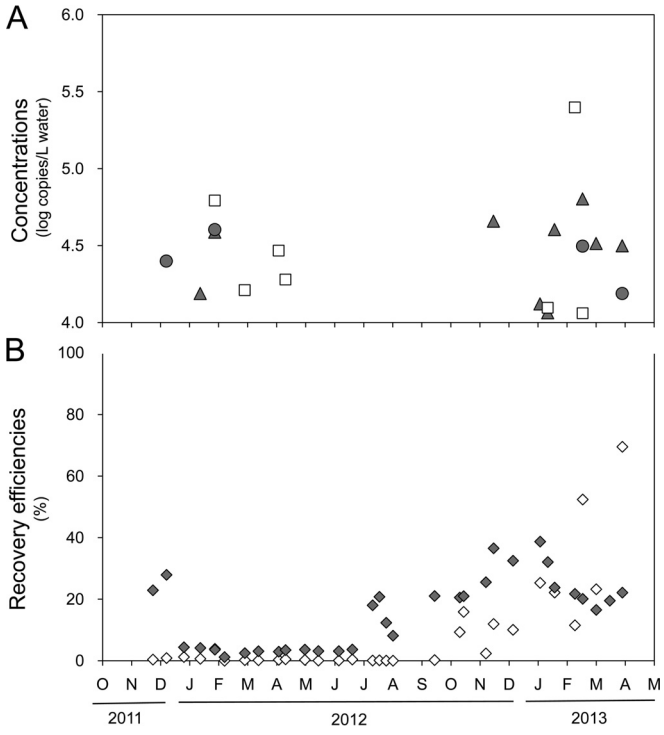

FIG 2 Environmental application of the MFQPCR system for viral quantification. (A) Concentrations of NoV GI (●), NoV GII (▲), and RoV (□) in river water samples collected from November 2011 to April 2013. (B) Recovery efficiencies of the sample process control (i.e., MNV) (◇) and internal amplification control (i.e., plasmid DNA containing the MgV gene) (◆).

In this study, we added plasmid DNA that contained MgV gene sequences to the cDNA samples prior to the STA reactions and quantified MgV signals by MFQPCR. This approach allows us to assess the occurrence of inhibition during STA and qPCRs but

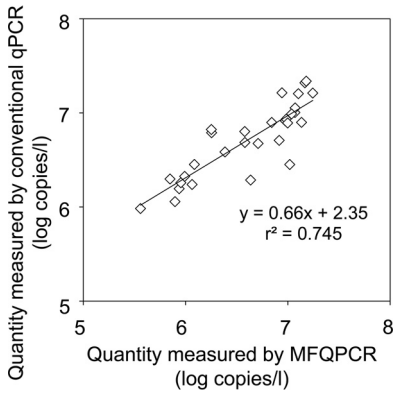

FIG 3 Correlation between concentrations measured by MFQPCR and those measured by conventional qPCR. Quantitative results for MNV were used for comparison. The linear regression equation and goodness-of-fit (r^2) value are also shown.

does not provide information on the occurrence of inhibition during cDNA synthesis. If we added a known amount of MgV or other control RNA to our RNA samples, we would be able to assess the occurrence of inhibition during cDNA synthesis. This should be done in the future.

Comparison with conventional qPCR assays. Conventional qPCR assays were used to verify the results obtained by MFQPCR. Similar to the MFQPCR results, NoV GI and GII and RoV were detected with similar concentration ranges in the water samples collected during the winter. Because the number of positive samples was small, we could not do a correlation analysis between MFQPCR and conventional qPCR results for NoV GI and GII and RoV. However, we found a strong correlation between MNV concentrations measured by MFQPCR and those measured by conventional qPCR ($r = 0.86$, $P < 0.01$) (Fig. 3). This suggested that the quantitative data obtained by MFQPCR were as accurate and reliable as those obtained by conventional qPCR, similar to the previously developed bacterium-targeting MFQPCR system (19).

Simultaneous detection of multiple pathogens was previously reported based on microarray (20), TaqMan arrays (22, 23), and other commercially available test kits (reviewed in reference 48). However, many of these methods do not provide quantitative information, which is essential for QMRA (49), or have limited throughput. Our MFQPCR approach can provide quantitative information on multiple pathogens, as accurate as that obtained by conventional qPCR, for many samples (up to 92 samples per run). In addition, the running cost for MFQPCR is \$0.17/assay/sample, including reagent cost for STA and qPCRs and the 96.96 chip but excluding costs for labor and equipment, which is less expensive than conventional qPCR (\$0.45/assay/sample). These characteristics of MFQPCR are advantageous for routine food and water quality monitoring and risk assessment (19). Although it is difficult to distinguish infectious and noninfectious viruses based on the PCR-based detection methods, including MFQPCR, several approaches, such as the use of propidium monoazide (50), enzymatic digestion of free nucleic acids (6, 51), or biotinylation followed by spin column separation of damaged viral particles (52), could overcome this problem. Combinations of these approaches and MFQPCR should be tested in the future to better predict microbial risks associated with water and other environmental samples.

Simultaneous quantification of pathogenic bacteria and viruses. The MFQPCR system developed in this study was run using the same conditions as the MFQPCR system that we previously developed to target bacterial pathogens (18). Thus, we could run bacterial MFQPCR and viral MFQPCR on the same chip. Simultaneous quantification of bacterial and viral pathogens was experimentally verified (data not shown); however, we needed to run STA reactions separately because the primer combinations for STA reactions were different for the bacterial and viral MFQPCR systems. In addition, the procedures used for DNA/RNA extraction were different for bacteria and viruses, and cDNA synthesis was required only for viral MFQPCR. To overcome these problems, we will need to optimize the primer combinations for STA amplification of both bacterial and viral DNA/cDNA and develop a simultaneous DNA/RNA extraction method for bacterial and viral samples. These are some of our future goals.

In conclusion, we developed an MFQPCR system for the simultaneous quantification of multiple pathogenic viruses. This MFQPCR system is applicable to monitoring viral pathogens in natural environmental water samples. Combined with our MFQPCR system for quantifying multiple bacterial pathogens (18), this method has great potential for routine water quality monitoring and QMRA.

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